ANTIBODIES TO THE PROPEPTIDE OF CANDIDA ALBICANS AND METHODS OF USE

FIELD OF THE INVENTION

[0001] The present invention relates in general to antibodies which can bind to the propeptide sequence of the Int1p protein of *Candida albicans* and methods of utilizing such antibodies to prevent and treat infections from microorganisms such as *C. albicans*, and in particular to agents and antibodies capable of disrupting the propeptide region or other subregions of the Int1p protein and the use of such agents and antibodies in the treatment and prevention of infection from yeasts such as *Candida albicans* and other microorganisms expressing the Int1p protein.

BACKGROUND OF THE INVENTION

[0002] The dimorphic yeast, *Candida albicans*, is the leading fungal pathogen in normal hosts and in patients with damaged immune systems. In normal hosts, disease caused by *C. albicans* ranges from mild, easily treated, superficial disease (e.g., thrush in newborn infants; paronychia in workers whose hands are immersed in water) to more severe, chronic or recurrent infections (e.g., candidal vaginitis). It is estimated that 5% of women of child-bearing age will suffer from recurrent candidal vaginitis (Hurley, *Proc. R. Soc. Med.* 70 (Suppl., 4), 1-8 (1970), and that virtually every woman will experience at least one episode during her reproductive years. Vaginitis is particularly frequent in otherwise normal females with diabetes or a history of prolonged antibiotic or oral contraceptive use. While short-term topical therapy is effective in treating individual episodes of vaginitis, such agents do not prevent recurrences. Thus, even in the normal host, infection with *C. albicans* can occur at epithelial surfaces, and recurrences are not prevented by presently available therapies.

[0003] In immunocompromised hosts such as cancer patients, transplant patients, post-operative surgical patients, premature newborns, or HIV-infected people, *C. albicans* ranks as the leading fungal pathogen. Invasion leading to systematic infection may also develop in neutropenic patients whose t-cell function is comprised. (Hostetter MK, *Clinical Microbiology Reviews,* Jan 1994, pp. 29-42.) In this population, disease ranges from aggressive local infections such as periodontitis, oral ulceration, or esophagitis in HIV-infected patients, to complex and potentially lethal infections of the bloodstream with subsequent dissemination to brain, eye, heart, liver, spleen, kidneys, or bone. Such grave prognoses require more toxic therapy, with attendant consequences from both the underlying infection and the treatment. Here again, the infection typically begins at an epithelial site, evades local defenses, and invades the bloodstream in the face of immunosuppression. Strategies to interrupt candidal adhesion therefore have broad applicability to the prevention of mild but recurrent disease in the normal host and to the reduction of substantial morbidity and mortality in the immunocompromised.

[0004] It is well recognized that *C. albicans* adheres to epithelial and endothelial cells in the human host, often times by recognizing proteins of the extracellular matrix called ligands. These ligands include proteins such as fibronectin, vitronectin, fibrinogen, the C3 degradation fragment iC3b, or the shorter C3 degradation fragment C3d. Because recognition of all of these proteins except C3d appears to be dependent upon the amino acid sequence ARGININE-GLYCINE-ASPARTIC ACID (or R-G-D), these candidal adhesions are thought to operate like the vertebrate integrins and are called "integrin-like proteins" or "integrin analogs."

[0005] Vertebrate integrins are composed of two subunits: an α-subunit and a β-subunit. There are approximately 14 α and 8 β subunits described to date in vertebrate cells. Using monoclonal or polyclonal antibodies to vertebrate integrins, several investigators have obtained evidence for integrin-like proteins in *C. albicans*.

[0006] One such protein is the protein Int1p of *Candida albicans*, and this protein has been observed to function as an adhesin, to participate in morphologic switching of blastospores to hyphae, and has been linked to virulence in mice. Rapid mortality ascribable to INT1/INT1 strains suggested that Int1p may have an immunomodulatory role. Pathogenesis studies using a mouse fungemia model have linked Int1p mediated adhesion and filamentation to *Candida albicans* virulence (Gale et al., *Science* 279:1355-1358, 1998), and intravenous inoculation of an *int1/int1* double disruption mutant (CAG3) is associated with reduced mortality and renal inflammation compared to the wild type INT1/INT1 strain (CAF2) (see Bendel et al., *Mol. Genetics and Metabolism* 67:343-351, 1999).

[0007] However, mortality rates from infections from organisms such as disseminated candidas remain high despite aggressive antifungal therapy (Todischini, *J. Intern Dis.* 1:S37-S41, 1997), and a highly effective method of treating or preventing diseases caused by *Candida albicans* and other similar microorganisms expressing Int1p has yet to be obtained.

SUMMARY OF THE INVENTION

[0008] It is thus an object of the present invention to provide a method of effectively treating infection caused by *Candida albicans* and other similar microorganisms which express the Int1p protein.

[0009] It is further an object of the present invention to provide a method of effectively treating or preventing infection caused by *Candida albicans*.

[0010] It is still further an object of the present invention to provide a method of isolating a propeptide and treating or preventing infection caused by *Candida albicans* through generation of antibodies against the propeptide.

[0011] It is even further an object of the present invention to isolate specific regions of the Int1p protein from *C. albicans* and other similar microorganisms such as *S. cerevisiae* which express the Int1p protein, and provide agents and antibodies capable of binding said regions.

[0012] It is even further an object of the present invention to isolate specific regions of the Int1p protein from *C. albicans* and other similar microorganisms such as *S. cerevisiae* which express the Int1p protein, and provide peptides or antibodies which can either disrupt the cleaving of the propeptide, or which can act to block the potential binding sites for the propeptide, namely the antigen-presenting cell or the T-lymphocyte binding region.

[0013] It is also an object of the present invention to provide a method of inhibiting the activity of the Int1p protein of *Candida albicans* so as to prevent or treat infections caused by microorganisms expressing the Int1p protein.

[0014] These and other objections are achieved by the present invention which comprises isolating a peptide from one of a number of specific regions from the Int1p protein of *C. albicans* and treating or preventing an infection from *C. albicans* or other microorganism expressing the Int1p protein by administering to a human or animal patient an effective amount of an antibody composition or other agent which can bind to those specific regions and thus disrupt the activity of the Int1p protein. In particular, the invention relates to the isolation of the propeptide of the Int1p protein and the development of antibodies or other agents which can bind to the propeptide and thus be useful in methods of disrupting the activity of the Int1p protein, such as by preventing the cleaving of the propeptide, and thus prevent or treat infections from *C. albicans* or other microorganisms expressing the Int1p protein. The invention also relates to the generation of peptides which can be used to block the binding of the superantigen to the antigen-presenting cells and/or the T-lymphocytes of the host so as to be useful in methods of preventing or treating infections from *C. albicans* or other microorganisms expressing the Int1p protein

BRIEF DESCRIPTION OF THE DRAWING FIGURES

[0015] Fig. 1 is a depiction of the amino acid sequence of the Int1p protein from *C. albicans*.

[0016] Figs. 2A and 2B show the nucleic acid sequence coding for the Int1p protein from *C. albicans*.

[0017] Fig. 3 is a schematic representation of the activation of a general proprotein convertase which shows the presence of a signal peptide, the propeptide, an inactive subtilisin and P-domain, and the manner of activation.

[0018] Fig. 4 is a schematic representation of the int1p protein as compared to a generic proprotein convertase which illustrates the clipping of the Int1p propeptide which is cleaved to become a superantigen at the same time the subtilisin regions are activated as well.

[0019] Fig. 5 shows the P Domain subtilisin motifs from a variety of proteins.

[0020] Fig. 6 shows a comparison of the high-affinity heparin binding site of *Mycobacterium tuberculosis* heparin-binding hemagglutin adhesin (HBHA) with the heparin-binding site of the Int1p protein of *Candida albicans*.

[0021] Fig. 7 depicts the activation of T lymphocytes after incubation with *INT1/INT1* blastospores (squares) or *int1/int1* blastospores (diamonds). Data from five normal adult donors are shown. *$p<0.05$.

[0022] Fig. 8 depicts the effects of antibodies against the MHC Class II determinant HLA-DR (black columns) on lymphocyte activation in response to PHA, TSST-1, *INT1/INT1 C. albicans*, or *int1/int1 C. albicans*. An irrelevant murine IgG (hatched bars) served as isotype control. *$p<0.04$.

[0023] Fig. 9 shows the effects of TSST-1, *INT1/INT1 C. albicans*, *int1/int1 C. albicans*, and phytohemagglutinin on stimulation of Vβ subsets. Unactivated T lymphocytes served as control. *$p<0.05$.

[0024] Fig. 10. is a schematic view showing the regions of a generic proprotein convertase.

[0025] Fig. 11 is a schematic representation of the Int1p peptide regions in accordance with the present invention including an identification of regions recognized by certain anti-peptide polyclonal antibodies.

[0026] Fig. 12 illustrates the flow cytometry of surface-exposed domains of Int1p when *C. albicans* blastospores are grown to exponential phase in the

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absence (left panel) or presence (right panel) of 2 units of heparin. X axis represents log-scale fluorescence; Y axis represents percent yeasts fluorescing. Hatched area - fluorescence with anti-INT600. Gray area-fluorescence with anti-CBS2. Fluorescence of *C. albicans* cells incubated with rabbit IgG serves as control - dotted line.

[0027] Fig. 13 is a Western blot of supernatants from *INT1*-expressing *S. cerevisiae* grown in the absence or presence of heparin and probed with rabbit polyclonal antibodies to the Int1p amino terminus (anti-INT600), to the second divalent cation binding site (anti-CBS2), or to the RGD domain (anti-RGD).

[0028] Fig. 14 are immunoblots showing the purification of Pep_{263}. Silver stain lanes 1-4. Western blot lanes 5 and 6. Lane 1 - *S. cerevisiae* lysate after expression of Pep_{263}; lane 2 - fraction 300-1 from nickel column; lane 3 - fraction 300-2; lane 4 - purification of Pep_{263} to homogeneity; Lane 5 shows that a single band of 44 kDa on silver strain (lane 4) reacted with anti-His antibody on Western blot.

[0029] Fig. 15 is a graphic representation of the percent of T lymphocytes up-regulating the IL-2 receptor (Y axis) in response to Pep_{263} presented as soluble antigen (leftmost group of three bars), as antigen bound to the plate (middle group), or as antigen bound to an anti-His antibody attached to protein A beads (right group).

[0030] Fig. 16 is a schematic representation of a model for the participation of Int1p in Candidemia.

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[0031] Fig. 17 shows the MHC-II Binding Sites in the Int1p protein, and in Mycoplasma arthritidis, as disclosed in *J. Exp. Med.* 183:1105-1110 (1996), incorporated herein by reference.

[0032] Fig. 18 shows the linkage of the T lymphocyte to the antigen-presenting cell through the superantigen which is produced after the propeptide is cleaved.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0033] In accordance with the present invention, the present inventors have now discovered and isolated several distinct regions of the Int1p protein, and the present invention is directed to treating or preventing infections from microorganisms which express the Int1p protein, including yeast of the *Candida* species such as *Candida albicans*, and other microorganisms such as *S. cerevisiae*, by disrupting the regions, including the propeptide region, which are involved with the pathways by which the Int1p protein is activated in a host. In addition, the present invention is directed to agents and antibodies which can bind to the specific regions of the Int1p protein and which thus can be useful in treating or preventing *C. albicans* infections. In general, it is desired to develop antibodies which can prevent the propeptide from cleaving, and/or antibodies that will bind to the propeptide and thus disrupt the activation of the Int1p protein.

[0034] In one of the preferred embodiments of the invention, the invention relates to peptides, either linear or cyclic, which have the same sequence as that of the sites on the superantigen propeptide which will bind to two sites, namely the antigen-presenting cell (such as the MHC-II locus) and the T lymphocytes on the host cell. In the Int1p protein, the MHC-II binding peptide appears to be in the region of from amino acid 239 through 254 (in the propeptide region of 1-263) of the sequence of the protein shown in Fig. 1, and this sequence is shown in Fig. 17. Accordingly, the use of this peptide, or other blocking peptides, is contemplated in accordance with the invention in any suitable form, e.g., pharmaceutically acceptable compositions, as would be used for administration to a human or animal patient. These types of blocking peptides can thus be administered to the host as a method of blocking the sites that would become bound to the superantigen propeptide, and thus can be used to prevent or treat infections caused by the Int1p protein.

[0035] In a further aspect of the present invention, it is contemplated that treatment or prevention of infections caused by microorganisms such as *C. albicans* may be achieved by causing mutations in the specific regions as set forth herein which can cause conformational or other changes to the peptides coded by these regions and thus disrupt the immunomodulatory ability of the Int1p protein. The gene sequence and the peptide sequence for the Int1p protein has previously been disclosed, e.g., in *Proc. Natl. Acad. Sci. U.S.A.* 93 (1), 357-361 (1996), incorporated herein by reference. In addition, further information regarding Int1p has been provided in pending U.S. patent application Ser. No. 09/264,604 and in U.S. Pat. No. 5, 886,151, both incorporated herein by reference. Finally, the amino acid sequence of the Int1p protein is shown in Figure 1, and the DNA sequence is shown in Figures 2a-2b. In the preferred embodiments, those mutations in accordance with the invention will be those which can prevent the cleaving of the propeptide, or which can disrupt the binding of the propeptide superantigen to the antigen-presenting cells or to the T lymphocytes of the host. Accordingly, in accordance with the invention, mutations in either or both of these propeptide binding regions are preferred.

[0036] As will be shown further below, the present invention thus relates to antibodies which can bind to the specific regions from the *C. albicans* Int1p protein as set forth below and the use of those antibodies in disrupting the *C.* albicans activity in human or animal hosts so as to prevent or treat infections caused by this or other similar microorganisms expressing the Int1p organism. Accordingly, in one embodiment, the present invention relates to isolated and/or purified antibodies, such as polyclonal or monoclonal antibodies, which have been generated against specific regions of the *C. albicans* Int1p protein which can be useful in methods of preventing and treating candidal and other yeast infections caused at least in part by the Int1p protein and its immunomodulatory ability. The term "antibodies" as used herein includes monoclonal, polyclonal, chimeric, single chain, bispecific, simianized, and humanized or primatized antibodies as well as Fab fragments, including the products of an Fab

immunoglobulin expression library, and generation of any of these types of antibodies or antibody fragments is well known to those skilled in the art. As indicated above, it is desirable to provide antibodies which can disrupt the activation of the Int1p protein in any of a number of ways, including preventing the cleaving of the propeptide, or disrupting the binding of the cleaved superantigen to host cells at its binding sites, namely the antigen-presenting cell (such as the MHC-II locus) or the superantigen-binding site on T lymphocytes. As described further below, these antibodies are preferably used in amounts effective to prevent or treat infections from *C. albicans* and other similar microorganisms, and these antibodies may be produced in any of a number of suitable ways well known in the field to produce polyclonal or monoclonal antibodies. For example, in addition to generating polyclonal antibodies by injecting the isolated peptides of the invention into a suitable animal model and isolating the antibodies therefrom, monoclonal antibodies directed to the Int1p regions described below may also be generated using the method of Kohler and Milstein (see, e.g., *Nature* 256:495-7, 1975), or other suitable ways known in the field. Antisera prepared using monoclonal or polyclonal antibodies in accordance with the invention are also contemplated and may be prepared in a number of suitable ways as would be recognized by one skilled in the art.

[0037] In addition, the invention relates to the use of agents which can bind to the specific regions below so as to disrupt these peptides and again inactivate the infectious and immunomodulatory pathways by which microorganisms expressing the Int1p protein by become virulent. Finally, it is also contemplated that mutations to these regions, whether to the amino acid sequences or to the nucleic acid sequences coding these peptides, may also be utilized in order to disrupt the functioning of the Int1p protein and to make the infectious microorganisms ineffective or less virulent.

[0038] In one embodiment of the present invention, the invention relates to the isolation of the propeptide of the Int1p protein and the use of this propeptide in generating antibodies and other agents which will be useful in the treatment or

prevention of *C. albicans* infection. This propeptide constitutes amino acids 1-263 of the Int1p protein, such as shown in Fig. 1, and has been identified as peptide Pep_{263}. As the present inventors have determined, the propeptide, Pep_{263} constitutes a superantigen-like moiety which is released from Int1p and which plays a major role in activating T lymphocytes in host cells. Accordingly, an antibody or other agent capable of binding to this propeptide can be utilized in a method of disrupting the activation of T lymphocytes caused by microorganisms such as *C. albicans* and *S. cerevisiae*, and thus can be utilized in methods of preventing, treating, or reducing the virulence of infections from such microorganisms which express Int1p. In particular, the antibody to the propeptide in accordance with the present invention will be able to disrupt the functioning of the Int1p protein, e.g., such as by binding the propeptide and/or preventing the cleaving of the propeptide and thus stopping the release of the propeptide in its superantigen form.

[0039] The propeptide Pep_{263} also contains a heparin binding site at amino acids 155-169, and it appears that activation of T lymphocytes is triggered by Pep_{263} when this peptide is cleaved from the amino terminus of Int1p in a reaction accelerated by physiologic doses of heparin. In the absence of heparin, Pep_{263} appears to be covert and is generally not detectable by antibodies such as anti-INT600, an antibody to the first 600 amino acids of the Int1p protein. However, in the presence of heparin, the amino terminus of Int1p is exposed, at which point Pep_{263} is cleaved and released into the fluid phase where it exhibits superantigen-like effects culminating in the release of pro-inflammatory cytokines that influence the clinical outcome and cause or enhance infection of microorganisms such as *C. albicans* and *S. cerevisiae* which express Int1p. Accordingly, agents and antibodies to Pep_{263} in accordance with the present invention can be useful in methods to prevent or treat infections in microorganisms expressing the Int1p protein and to eliminate or reduce the activation of T lymphocytes caused therefrom.

[0040] As shown in the schematic drawing Figs. 3 and 4, activation of "subtilisin-like" proprotein convertases occurs in the Int1p protein which ultimately leads to the cleaving of the propeptide and the activation of the virulent form of the microorganism. In Fig. 3, the schematic analysis of the Int1p protein shows the presence of a signal peptide, the propeptide, an inactive subtilisin and the P-domain. The processing or "P-domain" is employed to clip the propeptide at the carboxy terminal side of dibasic residues, thereby releasing the propeptide. Exposed D-H-N-S active site residues assume the subtilisin serine protease conformation. This amino terminal processing is shown further in Fig. 4 wherein the original form of Int1p is transformed by the clipping of the propeptide, which includes heparin binding region 155-169, and which is cleaved to become a superantigen at the same time the subtilisin regions are activated as well. P Domain subtilisin motifs from a variety of proteins are compared as shown in Fig. 5. Fig. 6 shows a comparison of the high-affinity heparin binding site of *Mycobacterium tuberculosis* heparin-binding hemagglutin adhesin (HBHA) with the heparin-binding site of the Int1p protein of *Candida albicans*.

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[0041] As thus has been shown by the present inventors, the specific regions of the Int1p protein which are involved in the activation of T lymphocytes by this protein all present target sites for disruption of infectivity and virulence of microorganisms that express this protein such as *C. albicans* and *S. cerevisiae*. As indicated above, the propeptide region at amino acids 1-263 which includes a heparin binding site is critical to the activation process in that this propeptide is cleaved from the protein in order to become a superantigen which has been shown to be able to immunomodulate host cells. In accordance with the invention, antibodies or other agents which can bind this region can thus be useful to prevent T-cell activation and can thus be employed in methods of preventing or treating outbreaks of infections from microorganisms expressing Int1p.

[0042] Still other specific regions utilized in the activation process have been identified, and these peptides can be isolated and/or purified so as to be used in

generating antibodies and other agents which will bind to these proteins or otherwise be able to disrupt the Int1p activation process. Included in these regions in addition to the propeptide region at amino acids 1-263 are the Catalytic domain 1 at amino acids 435-639, the Catalytic domain 2 at amino acids 738-949, and the Processing domain (or "P-domain") motif at amino acids 1022-1236, as best shown in Fig. 11. In accordance with the present invention, isolated and/or purified antibodies, produced for example in the manner described above, may be generated against the specific regions recited above, and effective amounts of said antibodies may be employed in methods of preventing or treating infections from *C. albicans* or other microorganisms that express the Int1p protein. Similarly, other methods of treatment or prevention in accordance with the present invention would include agents which bind to or otherwise disrupt these specific regions so as to reduce or eliminate Int1p activity, or mutations in these specific regions of wild-type sequences which also are effective in reducing or eliminating Int1p activity. As indicated above, in the most desirable embodiment, these antibodies will function so as to disrupt Int1p activity, such as by binding the peptide regions and/or preventing the cleaving of the propeptide and thus stopping the release of the propeptide in its superantigen form.

[0043] In addition, other peptides or antibodies which can disrupt the binding of the superantigen to the host cells are also provided in accordance with the invention. The superantigen enables the activation of T lymphocytes through a two-fold binding system wherein the superantigen binds to both the T cell and to the antigen-presenting cell, such as at the MHC Class II locus, such as shown in Fig. 18.

[0044] It is also contemplated that isolated nucleic acids coding for the regions set forth above, namely the propeptide region at amino acids 1-263, the catalytic domains 1 and 2, and the processing domain as shown in Fig. 11, will be contemplated in accordance with the present invention. As would be

recognized by one skilled in the art, nucleic acid sequences in accordance with the invention will include not only the specific regions of the nucleic acid sequence as shown in Figs. 2A-2B which correspond to the peptide regions as set forth above, but to any alternative nucleic acid sequences coding for those amino acid sequences. The isolated nucleic acids of the invention will be useful in many appropriate ways, including generating the peptide regions in accordance with the invention through recombinant means so that these recombinant peptides may be used to generate appropriate antibodies. In addition, it is contemplated that mutations to the peptide and nucleic acid sequences in these regions will also be useful in providing alternative methods by which to disrupt the Int1p activation pathways.

[0045] In addition to the use of agents and antibodies which bind to the propeptide and/or other specific regions of the Int1p protein as set forth above in methods of treating or preventing infection, the present invention also contemplates the use of these antibodies in a variety of ways, including the detection of the presence of microorganisms such as *C. albicans* or *S. cerevisiae* and thus using antibodies to diagnose infections caused by microorganisms expressing Int1p, whether in a patient or in medical materials which may also become infected, is contemplated in accordance with the invention. For example, one such method of detecting the presence of infections by microorganisms expressing Int1p involves the steps of obtaining a sample suspected of being infected, and lysing the cells so that the DNA can be extracted, precipitated and amplified. Following isolation of the sample, diagnostic assays utilizing the antibodies of the present invention may be carried out to detect the present of Int1p microorganisms such as *C. albicans* or *S. cerevisiae*, and such assay techniques for determining such presence in a sample are well known to those skilled in the art and include methods such as radioimmunoasssay, Western blot analysis and ELISA assays.

[0046] Accordingly, antibodies in accordance with the invention may be used for the specific detection of Int1p-producing microorganisms, for the prevention or

treatment of infection from said microorganisms, or for use as research tools. As indicated above, the term "antibodies" as used herein includes monoclonal, polyclonal, chimeric, single chain, bispecific, simianized, and humanized or primatized antibodies as well as Fab fragments, including the products of an Fab immunoglobulin expression library. Generation of any of these types of antibodies or antibody fragments is well known to those skilled in the art.

[0047] As would also be recognized by one skilled in the art, the antibodies of the present invention may also be formed into suitable pharmaceutical compositions for administration to a human or animal patient in order to treat or prevent an infection caused by yeast such as *C. albicans* or *S. cerevisiae*. Pharmaceutical compositions containing the antibodies of the present invention, or effective fragments thereof, may be formulated in combination with any suitable pharmaceutical vehicle, excipient or carrier that would commonly be used in this art, including such as saline, dextrose, water, glycerol, ethanol, other therapeutic compounds, and combinations thereof. As one skilled in this art would recognize, the particular vehicle, excipient or carrier used will vary depending on the patient and the patient's condition, and a variety of modes of administration would be suitable for the compositions of the invention, as would be recognized by one of ordinary skill in this art. Suitable methods of administration of any pharmaceutical composition disclosed in this application include, but are not limited to, topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal and intradermal administration.

[0048] For topical administration, the composition is formulated in the form of an ointment, cream, gel, lotion, drops or solution. Alternatively, when so desired, wound or surgical dressings, sutures and aerosols may be impregnated with the composition to further prevent infection. The composition may contain conventional additives, such as preservatives, solvents to promote penetration, and emollients. Topical formulations may also contain conventional carriers such as cream or ointment bases, ethanol, or oleyl alcohol.

[0049] Still further, the isolated antibodies of the present invention, or active fragments or portions as set forth above, may also be utilized in the development of vaccines for passive or active immunization against candidal-type infections or other infections associated with Int1p-producing microorganisms. Further, these compositions may also be administered to a wound or used to coat medical devices or polymeric biomaterials *in vitro* and *in vivo*. In addition, the antibody may be modified as necessary so that, in certain instances, it is less immunogenic in the patient to whom it is administered. For example, if the patient is a human, the antibody may be "humanized" by transplanting the complimentary determining regions of the hybridoma-derived antibody into a human monoclonal antibody as described, e.g., by Jones *et al.*, *Nature* 321:522-525 (1986) or Tempest *et al. Biotechnology* 9:266-273 (1991).

[0050] In one embodiment, the isolated peptides in accordance with the invention may be used in the preparation of a vaccine which comprises one or more of the Int1p peptides as described above in an amount sufficient to generate an immunological response. In addition, antibodies in accordance with the invention may be used as a passive vaccine which will be useful in providing suitable antibodies to treat or prevent candidal or other similar infections. As would be recognized by one skilled in this art, a vaccine may be packaged for administration in a number of suitable ways, such as by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration or nasopharyngeal (i.e., intranasal) administration. Although many methods of administering the vaccine will be suitable, the particular mode of administration will depend on the nature of the infection to be dealt with and the condition of the patient. The vaccine is preferably combined with a pharmaceutically acceptable carrier to facilitate administration, and the carrier may be include common materials such as water or a buffered saline, with or without a preservative. The vaccine may be lyophilized for resuspension at the time of administration or in solution.

[0051] The preferred dose for administration of an antibody composition in accordance with the present invention is that amount will be effective in

preventing of treating a yeast infection or infection from other microorganisms that express the Int1p protein. As one skilled in the art would recognize, such an effective amount will vary greatly depending on the nature of the infection and the condition of a patient. As indicated above, an "effective amount" of antibody or pharmaceutical agent to be used in accordance with the invention is intended to mean a nontoxic but sufficient amount of the agent, such that the desired prophylactic or therapeutic effect is produced. The exact amount of the antibody or a particular agent that is required will thus vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, the particular carrier or adjuvant being used and its mode of administration, and the like. Accordingly, the "effective amount" of any particular antibody composition will vary based on the particular circumstances. However, an appropriate effective amount may be determined in each case of application by one of ordinary skill in the art using only routine experimentation. The dose should be adjusted to suit the individual to whom the composition is administered and will vary with age, weight and metabolism of the individual. The compositions may additionally contain stabilizers or pharmaceutically acceptable preservatives, such as thimerosal (ethyl(2-mercaptobenzoate-S)mercury sodium salt) (Sigma Chemical Company, St. Louis, MO).

[0052] In another embodiment of the present invention, a kit which may be useful in isolating and identifying infections caused by microorganisms expressing Int1p which comprises the antibodies of the present invention in a suitable form, such as lyophilized in a single vessel which then becomes active by addition of an aqueous sample suspected of being infected with *C. albicans* or other similar microorganism. Such a kit will typically include a suitable container for housing the antibodies in a suitable form along with a suitable immunodetection reagent which will allow identification of complexes binding to the specific regions of the Int1p protein as set forth above. For example, the immunodetection reagent may comprise a suitable detectable signal or label,

such as a biotin or enzyme that produces a detectable color, etc., which normally may be linked to the antibody or which can be utilized in other suitable ways so as to provide a detectable result when the antibody binds to the antigen. Additionally, a method of identifying or diagnosing an infection of *C. albicans* or other microorganism expressing the Int1p protein is also provided wherein one or more antibodies to the peptide regions set forth above from the Int1p protein are introduced into a sample thought to be infected with a microorganism expressing Int1p, and the identification or diagnosis of the infection can be confirmed if binding to the sample is observed. Such binding can be observed in any of a number of suitable ways commonly used in the art, including, e.g., detectable labels, as described above.

[0053] In summary, the present invention thus provides isolated and/or purified regions of the Int1p protein which have been shown to be involved in pathways of activation which results in the virulent spread of microorganisms expressing Int1p, and also provides antibodies, antisera, and other agents which can bind to these specific regions, and/or which can disrupt the process of Int1p activation in other ways. Such antibodies and agents can therefore be utilized in effective methods of treating or preventing infections from microorganisms such as *C. albicans* or *S. cerevisiae* which express the int1p protein.

EXAMPLES

[0054] The following examples are provided which exemplify aspects of the preferred embodiments of the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: INVESTIGATIONS OF THE INT1P PROTEIN.

[0055] The protein Int1p of *Candida albicans* functions as an adhesin, participates in morphologic switching of blastospores to hyphae, and is linked to virulence in mice. Rapid mortality ascribable to INT1/INT1 strains suggested that Int1p may have an immunomodulatory role. Therefore, we investigated whether expression of Int1p on the surface of *C. albicans* influenced T cell activation.

[0056] *C. albicans* strains used in the Investigations:

[0057] CAF2 *INT1/INT1 URA3/ura3 (supplied by WA Fonzi, Georgetown University, Washington D.C.)*

[0058] CAG1 *INT1/int1 URA3/ura3 (see Gale et al., Science* 279:1355-1358, 1998)

[0059] CAG3 *int1/int1 URA3/ura3 (see Gale et al., Science* 279:1355-1358, 1998)

[0060] HLC-54 *INT1/INT1 URA3/ura3 cph1/cph1 efg1/efg1 (supplied by JR Kohler, Whitehead Institute, MIT, Cambridge, MA).*

Culture Conditions:

[0061] Blastospores grown to mid-exponential phase in YPD medium at 30°C shaking, were washed in PBS containing the subinhibitory dose of 0.2 μg/ml amphotericin B (Al-Bassam et al., *J. Antimicrob Chemother*, 15:263-269, 1985). Yeast were adhered to the bottom of 96 well culture plates by incubating 500,000 cells/well for 45 minutes at 37°C.

[0062] Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-hypaque centrifugation of heparinized blood. Washed PBMCs were suspended in RPMI1640 supplemented with 10% human AB serum, L-glutamine, sodium

pyruvate, nonessential amino acids and 0.2 μg/ml amphotericin B to maintain *Candida albicans* in the blastospores stage.

[0063] Cocultures were initiated by adding 200λ PBMCs at 2.5×10^6 cells/ml to the adherent blastospores upon removal of PBS/amphotericin B. Cultures were incubated at 37°C 5% CO_2 for 1-7 days. Control wells containing PBMC ± superantigen TSST-1 and the mitogen PHA were also established. The effect of MHC class II inhibition was assessed by adding anti-HLA-DR antibody to PBMCs prior to coculture. Where appropriate, CD3 cells were isolated to >97% purity using cell separation columns. Antigen presenting cells (APC) expressing MHC class II were isolated by plastic adherence. The antigen processing ability of APCs was abolished by pretreatment with 0.3% paraformaldehyde.

Flow Cytometry:

[0064] Cultures harvested at appropriate time points were stained for 10 minutes at RT with PE and FITC conjugated monoclonal antibodies to IL2R and CD3, respectively, to assess T cell activation. Similarly, T cell subsets CD4 and CD8 as well as $V_{beta}2$ and $V_{beta}8$ were analyzed by using Cychrome and PE labelled Mabs, respectively. Cells were quantitated by flow cytometry using FACS Vantage. (BD Biosciences, San Jose, CA.) Data analysis was performed using WinMD1 version 2.8 software. (Kindly supplied by Dr. Joseph Trotter, Scripps Research Institute, LaJolla, CA.)

Methods:

[0065] PBMCs from five normal healthy volunteers were cocultured with either CAF2 *INT1/INT1* or CAG3 *int1/int1* blastospores for days 1 through 7. IL2 receptor positive cells among the CD3 positive population indicate the frequency of activated T cells at each time point. Tests on five individuals showed that by day 4, the frequency of activated T cells was significantly greater for CAF2 cocultures. Clusters of activated T cells were also predominant by day 4 of

PBMCs cocultured with *INT1/INT1* blastospores. Under similar experimental conditions, *int1/int1* blastospores do not induce these T cell activation clusters.

[0066] PBMCs from a single donor were cultured alone or with *C. albicans* strains CAF2, CAG1, CAG3 or HLC-54 for five days. Only strain CAG3, the *int1/int1* null mutant failed to activate T cells above the level of unstimulated control.

[0067] PBMCs were cultured with either 10 μg/ml PHA, 4 μg/ml TSST-1 or 500,000 blastospores of either CAF2 or CAG3. Each culture condition was either left untreated or incubated with 10 μg/ml of either anti-HLA-DR antibody or an isotype control. CD3 positive cells were analyzed for IL2 receptor upregulation by flow cytometry. T cell activation induced by PHA was unaffected by anti-HLA-DR antibody as anticipated since mitogen activation is independent of MHC class II. However, the response to superantigen TSST-1 was significantly inhibited since binding to $V_{beta}2$ of the TCR and the beta chain of class II is required for activation. Anti-HLA-DR antibody significantly depressed the upregulation of IL2R on T cells cocultured with CAF2 *INT1/INT1* but not with CAG3 *int1/int1* suggesting a role for MHC class II molecules in Int1p-mediated T cell activation.

[0068] Cultures as described above were expanded during the last 24 hours of culture with human IL2. Cultures were analyzed by flow cytometry for the expansion of either T cell $V_{beta}2$ or $V_{beta}8$ subsets. Significant expansion of the $V_{beta}2$ subset occurred in activations with the $V_{beta}2$ specific superantigen TSST-1 as well as with blastospores of CAF2 but not with CAG3. The frequency of $V_{beta}8$ T cells was similar to the unactivated control for all conditions. Thus, there is preferential expansion of at least the $V_{beta}2$ subset by *C. albicans* expressing Int1p.

[0069] PBMCs that were cocultured with either CAF2 *INT1/INT1* or CAG3 *int1/int1* blastospores were tested to determine frequencies of CD4 and CD8 T cells by flow cytometry. The ratio of CD4:CD8 cells was <1:1 for T cells expanded by CAF2 *INT1/INT1*, whereas all other activation conditions had ratios >1:1. CAF2 modulation of the CD4:CD8 ratio which was evident in the $V_{beta}2$ T

cell subset of one of the donors evidences a role for Int1p in activation-induced CD4 T cell loss.

[0070] T cells ($2.5X10^5$) were cultured with either 500,000 CAF2 blastospores or 4 μg/ml TSST-1 in the presence of APC ($2.5X10^5$) pretreated with or without 0.3% paraformaldehyde. In this case, T cell activation by CAF2 *INT1/INT1* blastospores occurred despite the inability of MHC class II expressing APCs to process antigen. Similarly, activation by TSST-1 was not inhibited by paraformaldehyde fixation of APC.

[0071] In summary, equal numbers of *C. albicans* blastospores from strains CAF2 INT1/INT1 and CAG3 int1/int1 in mid-exponential phase were incubated with PBMCs isolated from 5 normal healthy volunteers. IL2 receptor upregulation on CD3 cells was monitored by flow cytometry. The percentage of CD3 cells expressing IL2 receptor was significantly greater for activations using CAF2 blastospores (42.0 +/- 6.3) than for CAG3 (3.8 +/- 1.0), p=0.005. Antibodies to HLA-DR inhibited IL2 receptor expression on T cells activated by CAF2, but had no effect on T cells activated by CAG3. Preliminary data indicated a preference for $V_{beta}2$ T cell activation by CAF2. Analysis of CD4:CD8 subsets revealed that T cells activated by CAG3 blastospores had a normal CD4:CD8 ratio (2:1) similar to control T cells expanded with human IL2. However, after incubation with Int1p-expressing CAF2 blastospores, the CD4:CD8 ratio was depressed (<1:1) due to a reduction in the number of CD4 T cells. Thus, activation of a specific V_{beta} subset of T lymphocytes and depletion of CD4 cells are two mechanisms by which Int1p-bearing *C. albicans* modulate the cell-mediated immune response.

[0072] As a result of the above experiments, the following conclusions can be drawn:

[0073] *Candida albicans* blastospores expressing the protein Int1p activate human T lymphocytes whereas blastospores lacking Int1p surface expression do not.

[0074] Inhibition of IL2 receptor upregulation by anti-HLA-DR antibody indicates a dependence for MHC class II in Int1p induced T cell activation.

[0075] T cell activation by Int1p expressing blastospores is independent of antigen processing as indicated by resistance to APC paraformaldehyde fixation.

[0076] *Candida albicans* expressing Int1p preferentially activate the $V_{beta}2$ T cell subset.

[0077] Activation induced deletion of CD4 T cells by Int1p is a mechanism by which *Candida albicans* can modify the host immune response.

[0078] EXAMPLE 2: ADDITIONAL INVESTIGATIONS OF THE INT1P PROTEIN AND THE ISOLATION OF THE PROPEPTIDE FROM CANDIDA ALBICANS

INT1 Gene in *Candida albicans* and its Importance in Pathogenesis

[0079] Some years ago, we identified the *Candida albicans* gene *INT1*, which encodes a protein of Mr 188 kDa that mediates adhesion, medium-dependent filamentation, and virulence. See, e.g., Gale et al. *PNAS* 93:357-61 (1996); Gale et al. *Science* 279:1355-58 (1998), incorporated herein by reference. In particular, ICR mice given a tail vein injection of 10^5 wild type *C. albicans* expressing both *INT1* alleles (genotype *INT1/INT1*) showed 100% mortality by day eleven, while 90% of mice given a homozygous double disruptant (genotype *INT1/int1*) survived. Animals given a heterozygous mutant (genotype *INT1/int1*) or a re-integrant (genotype *int1/int1/INT1*) had intermediate mortality (40% survival). All strains replicated equally well and underwent filamentous growth in serum, and no differences in CFU in blood, kidney, or liver were found (36). Thus, defects in replication, filamentation, or organ dissemination did not explain *INT1*-dependent mortality.

INT1-Expressing *C. albicans* Activate T Lymphocytes, but the *int1* Knockout Strain Does Not

[0080] We therefore asked, "Why is the presence of *INT1* associated with death in mice?" Unlike other fungi, *C. albicans* does not produce mycotoxins, but

based on the available evidence, we considered the possibility that the encoded protein Int1p, or some part of it, might be a superantigen.

[0081] Peripheral blood mononuclear cells (PBMC's) from five normal donors were obtained by Ficoll-Hypaque centrifugation, suspended in RPMI 1640 supplemented with 10% human AB serum, L-glutamine, sodium pyruvate, non-essential amino acids, and 0.2 μg/ml amphotericin B to prevent germ tube formation. See Figure 7. PBMC's were incubated at 37°C in 5% CO_2 with 10^5 blastospores of *INT1/INT1 C. albicans* or an equal number of *int1/int1 C. albicans* (homozygous double disruptant) for one to seven days (n=7 expts.) The superantigen TSST-1 (800 μg/well) or the mitrogen PHA served as controls. Two color flow cytometry was used to plot the percentage of CD3 positive cells that expressed the IL-2 receptor (CD25).

[0082] Results: Although no significant activation of T lymphocytes was observed for the first three days of culture, on days four through seven, PBMC's incubated with *INT1/INT1 C. albicans* showed a significant increase in expression of the IL-2 receptor (CD25) on CD3+ cells, a marker for lymphocyte activation. PBMC's form the same donors did not increase expression of the IL-2 receptor when incubated with the *int1/int1* double disruptant. These results indicate that Int1p is required for activation of T lymphocytes by *C. albicans*.

[0083] Activated T lymphocytes were predominantly of the CD4 subset and were eliminated within 7-10 days after co-culture. In all donors, the CD4/CD8 ratio, which ranged from 1.8:1 to 2.2:1 on day 3, was reversed by day 7.

INT1-Associated Activation of T Lymphocytes Can Be Blocked by Antibodies to MHC Class II

[0084] PBMC's were pre-incubated with a monoclonal antibody to HLA-DR prior to stimulation with the mitogen PHA, the superantigen TSST-1, *INT1/INT1 C. albicans*, or *int1/int1 C. albicans*. T cell activation (up-regulation of the IL-2

receptor CD25) was measured by two-color flow cytometry and plotted on the Y-axis. See Figure 8.

[0085] Results: Antibodies against MHC Class II HLA-DR significantly inhibited T lymphocyte activation induced by toxic shock toxin TSST-1 and *INT1/INT1 C. albicans*. Anti-HLA-DR antibodies did not block T lymphocyte activation induced by the T cell mitogen PHA (which does not require antigen-presenting cells for its effects) or by *int1/int1 C. albicans*. These results confirm the participation of MHC Class II in the activation response of T lymphocytes stimulated by TSST-1 or *INT1/INT1 C. albicans*.

T Lymphocyte Activation in Response to Int1p Does Not Require Antigen Processing or Presentation by Antigen-Presenting Cells (APC's)

[0086] APC's were separated from PBMC's by a glass wool column and pre-treated with 0.3% paraformaldehyde (PFA) before being returned to co-culture with lymphocytes. TSST-1 and *INT1/INT1 C. albicans* were used as stimuli. In the absence of PFA, 44% of T lymphocytes were activated with *INT1/INT1 C. albicans* as stimulus; in the presence of PFA, 43% were activated. With TSST-1 as stimulus, 60% of T lymphocytes were activated in the absence of PFA treatment of APC's; in the presence of PFA, 56% were activated. Thus, PFA treatment did not inhibit T lymphocyte activation in response to TSST-1 or to *INT1/INT1 C. albicans*. When leupeptin and pepstatin were used to inhibit antigen processing and presentation, respectively, lymphocyte activation in response to TSST-1 and *INT1/INT1 C. albicans* was not inhibited. These results show that lymphocyte activation by Int1p is independent of antigen processing and presentation by APC's.

Expansion of Identical Vβ Subsets by the Soluble Superantigen TSST-1 and Int1p

[0087] The expansion of Vβ subsets was measured after stimulation of PBMC's with TSST-1 (800 μg), with *INT1/INT1 C. albicans*, with *int1/int1 C. albicans*, and with PHA. Unactivated PBMC's served as control (Figure 9).

[0088] <u>Results:</u> Incubation of PBMC's with wild type *C. albicans (INT1/INT1)* or TSST-1 preferentially expanded the Vβ2 subset of T-lymphocytes (black bars) but not the Vβ8 subset (open bars). There was no significant expansion of the Vβ2 subset when lymphocytes were incubated with *int1/int1 C. albicans* (double disruptant) or with phytohemagglutin. These preliminary experiments show Vβ subset specificity in response to TSST-1 and Int1p; comparing *INT1*$^{+}$ strains and *int1*$^{-}$ strains confirms that expansion of the Vβ2 subset was *INT1*-dependent.

<u>Release of Pro-Inflammatory Cytokines</u>

[0089] Peripheral blood mononuclear cells were stimulated with $5x10^5$ *INT1/INT1 C. albicans* blastospores, and production of TNFα, IL-6 and IL-4 was measured in supernatants on days 2, 4 and 6. As can be seen from the table below, *INT1/INT1 C. albicans* induce a predominantly Th1 response *in vitro* with elevations in TNFα and IL-6 that are comparable to those induced by staphylococcal enterotoxin B (SEB), a well-characterized superantigen. There is virtually no production of IL-4 in response to *C. albicans* or SEB. Interestingly, the TNFα response to *C. albicans* showed a near 40-fold variance (high responder = 7014 pg/ml on day 2; low responder = 165 pg/ml on day 2), while the response to SEB did not differ significantly in these two donors. Although one reason for the more consistent response to SEB could be its use as a soluble protein, another possible interpretation is that the response to *C. albicans* involves different MHC Class II alleles, different Vβ subsets, or differing kinetics of T cell activation and apoptosis. Hypothesis Two will address this possibility.

PBMC's FROM	TNFα (pg/ml)			IL-6 (pg/ml)			IL-4 (pg/ml)		
HIGH	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6

RESPONDER									
INT1$^+$*C. albicans*	7014	746	841	10109	8726	19764	<5	<5	<5
Staphylococcal enterotoxin	2143	631	102	3023	2459	2016	50	22	12
LOW RESPONDER	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
INT1$^+$ *C albicans*	165	20	ND	7660	6820	ND	<5	<5	<5
Staphylococcal enterotoxin	1963	667	ND	7850	7945	ND	89	20	ND

Identical Effects with *Saccharomyces cerevisiae* Expressing *INT1*

[0090] In order to assess these effects apart from other candidal antigens, we expressed *INT1* in *S. cerevisiae* YPH500 under the control of a galactose-inducible promoter. *INT1* was ligated into plasmid pBM272 for transformation of *S. cerevisiae* YPH 500; the resultant plasmid was named pCG01. Expression of Int1p was induced with 2% galactose. *S. cerevisiae* transformed with pBM272 served as control. Approximately 25% of donor PBMC's were activated after co-culture with *S. cerevisiae* expressing *INT1*, as measured by up-regulation of the IL-2 receptor on flow cytometry; no up-regulation occurred after co-culture with *S. cerevisiae* transformed with vector alone. These effects could be blocked by antibodies to HLA-DR. The Vβ2 subset was preferentially expanded by *S. cerevisiae* expressing *INT1*; no expansion of Vβ2 or Vβ8 subsets was noted in response to *S. cerevisiae* transformed with vector alone.

EFFECTS of Int1p PROTEIN in *C. ALBICANS* or *S. CEREVISIAE*	
X	Activates T lymphocytes, up-regulates IL-2 receptor, and releases pro-inflammatory cytokines
X	Requires antigen-presenting cells (APC's) for co-stimulatory molecules but not for antigen presentation or processing
X	Expands particular Vβ subsets

[0091] These experiments indicated that the presence of the protein Int1p was associated with superantigen-like effects, both in wild type *C. albicans* and in transformed *S. cerevisiae*. These are imperfect experiments because they compare a soluble superantigen (TSST-1) with either *INT1*$^+$ or *int1*$^-$ strains of *C.*

albicans; nevertheless, the results provide some indication that superantigen-like effects were associated with *INT1*$^{+}$ *C. albicans*. However, because most superantigens are secreted proteins of 22-29 kDa, and Int1p has a predicted mass of 188 kDa in its unglycosylated form, we hypothesized that limited proteolysis of Int1p might generate a soluble polypeptide that could serve as a superantigen.

[0092] As a potential mechanism for proteolysis, we considered the possibility that Int1p, like MMTV, might be cleaved by a proprotein convertase. A subset of serine endopeptidases, proprotein convertases cleave proproteins, or zymogens, to their active fragments by limited proteolysis at one or at most two specific cleave sites. In eukaryotes, these enzymes are called "subtilisin-like proprotein convertases" or SPC's. Most SPC's are autocatalytic and must be activated by cleavage of their propeptide before they can cleave their specific substrates. A model of a proprotein convertase is provided in Figure 10, and the canonical cleavage site is indicated with an arrow.

[0093] Most proprotein convertases exhibit several highly conserved features including a propeptide domain, distinguished by a canonical cleavage site just C-terminal to a pair of dibasic amino acids, most frequently KR or KK. A catalytic domain spans approximately 330 amino acids with an active site sequent of D-H-N-S [Asp-His-Asn-Ser], in which the initiating D is followed by a DX. This DDX motif has been shown in other systems (e.g., integrins) to be a recognition site for the binding of the RGD tripeptide; however, this interaction has never been explored with proprotein convertases. Catalytic domains may occur singly or in tandem. Lastly, a processing domain (or P-domain) also contains a D-H-N-S motif, but in six of the seven know SPC's, an RGD tripeptide is intercalated between the N and the S. The RGD motif is essential for cleavage of the propeptide; site-directed mutagenesis of the RGD tripeptide inhibits zymogen processing and mis-directs cellular trafficking of the unprocessed protein.

[0094] In Figure 11, a comparison of the Int1p sequence in *C. albicans* with the motifs essential for the proprotein convertases is shown, and this analysis

disclosed several sites of interest, including a dibasic cleavage site at residue 263, two putative catalytic domains, and an RGD sequence correctly situation in a possible P domain. Regions recognized by specific rabbit anti-peptide polyclonal antibodies developed in our laboratory as shown in brackets.

Identification of an Amino-Terminal Peptide from Int1p in Activating Supernatants

[0095] Subsequent experiments showed that the supernatants from exponentially replicating *INT1/INT1 C. albicans* or *INT1*-expressing *S. cerevisiae* were just as active as yeast cells in activating T lymphocytes, and all activity was contained in a pool of proteins weighing less than 50 kDa. Indeed, as little as 500 pmoles of supernatant proteins served to activate T lymphocytes.

Exposure of a Covert Amino-Terminal Domain in Int1p in the Presence of Heparin

[0096] Because colonization with *C. albicans* in the gastrointestinal tract does not induce superantigen-like effects, we hypothesized that some environmental factor relevant to fungemia might accelerate release of these <50 kDa amino-terminal polypeptides from Int1p. Heparin is known to enhance autocatalysis and to accelerate cleavage of a propeptide from a zymogen. These effects suggested that heparin might accelerate release of Int1p-derived fragments that might serve as superantigens. This possibility was all the more meaningful because heparin is an ever-present infusate in patients with intravascular catheters.

[0097] For these experiments, *INT1/INT1 C. albicans* blastospores were incubated in the absence or presence of heparin; flow cytometry with polyclonal antibodies to the second divalent cation binding site (anti-CBS2) or to the first 600 amino acids of the amino terminus (anti-INT600) was used to detect the

appearance of these domains (see Figure 11 for domains recognized by these antibodies).

[0098] Results: In the absence of heparin (Figure 12 - left panel), *C. albicans* blastospores displayed intensity fluorescence with anti-CBS2 (gray area) but did not fluoresce with anti-INT600. However with the addition of heparin, substantial fluorescence with anti-INT600 was now detectable (right panel). These results suggested that heparin exposed a covert amino-terminal domain and made it accessible for cleavage. If we were correct then a cleaved amino-terminal fragment of Int1p should be found in the supernatants of organisms grown in the presence of heparin but not in its absence.

Absence of Int1p Amino-Terminal Fragments in Culture Supernatants is Accelerated by Heparin

[0099] Figure 13 is a Western blot of supernatants from *INT1*-expressing *S. cerevisiae* grown in the absence or presence of heparin and probed with rabbit polyclonal antibodies to the Int1p amino terminus (anti-INT600), to the second divalent cation binding site (anti-CBS2), or to the RGD domain (anti-RGD).

[00100] Results: Supernatants probed with anti-CBS2 (panel 2) or anti-RGD (panel 3) showed identical banding patterns in the absence or presence of heparin. However, probing the supernatants with anti-INT600 disclosed two novel fragments of 27 kDa and 44 kDa only in the supernatants of organisms grown in the presence of heparin for three hours. In the absence of heparin, these fragments appeared at much reduced levels after 18 hours or more. Thus, heparin accelerated the appearance of two Int1p amino-terminal polypeptides in the supernatant. Supernatant containing the 27 and 44 kDa fragments activated T lymphocytes, while the other supernatants did not.

Localization of the Int1p Superantigen-like Fragment

[00101] From the foregoing experiments we had circumstantial evidence that amino-terminal fragments of Int1p (Mr 27 or 44 kDa) could be exposed in the presence of heparin, cleaved, and detected in the supernatant of *INT1*-expressing *S. cerevisiae*. If heparin accelerated the cleavage of Int1p by a proprotein convertase as occurs with the superantigen vSAG7 from MMTV, then an amino-terminal fragment encompassing 263 amino acids should be released from Int1p (Figure 11). A preliminary estimate of the mass of the first 263 amino acids of Int1p was 35 kDa, rising to 42 kDa is glycosylated. We therefore tested the possibility that the first 263 amino acids of Int1p, hereinafter called Pep_{263}, constituted the superantigen-like moiety.

[00102] In order to obtain direct evidence that Pep_{263} was responsible for the superantigen-like effects observed with *INT1/INT1 C. albicans* and *INT1*-expressing *S. cerevisiae*, we expressed Pep_{263} as a recombinant, His-tagged protein in *S. cerevisiae* and assessed its effects on T lymphocyte activation and expansion of Vβ subsets. *S. cerevisiae* was preferable to *E. coli* for expression in order to avoid the activating effects of lipopylsaccharide. *C. albicans* genomic DNA encoding amino acids 1 to 263 of Int1p was amplified by PCR and ligated in-frame to a 6X-His tage at the 3' end. This construct was inserted as a *Bam*HI/*Sal*I fragment into pBM272 and expressed from a galactose-inducible promoter in *S. cerevisiae* BJ3501, a protease-deficient strain. The His-tagged fusion protein appeared in the lysate (Figure 14, lane 1). *S. cerevisiae* lysate was chromatographed on a nickel column, and an anti-His Mab was used in a dotblot to detect the His-tagged protein as it was eluted from a nickel column by an imidazole gradient (0-500 mM imidazole). The His-tagged Pep_{263} eluted at a concentration of 300 mM imidazole in fractions 300-1 and 300-2 (Figure 14, lanes 2 and 3). An additional filtration step yielded a single band of 44 kDa on silver strain (Figure 14, lane 4), which reacted with anti-His antibody on Western

(Figure 14, lane 5). The use of these techniques has produced microgram quantities of the putative superantigen.

Activation of T Lymphocytes by Recombinant Pep_{263}

[00103] 100 picograms of Pep_{263} was then incubated with $5x10^6$ PBMC's in each of three reactions: (a) purified Pep_{263} as a soluble peptide; (b) purified Pep_{263} bound to the bottom of the tissue-culture well; (c) purified Pep_{263} immobilized by anti-His antibodies covalently linked to Protein A Sepharose beads. Fractions which also eluted at 300 mM imidazole but contained no His-tagged protein were used as control.

[00104] Results: Unactivated T lymphocytes (left-most bar in each group of three) or T cells incubated with eluted peptides that did not contain the His tag (middle bar in each group of three) did not upregulate the IL-2 receptor. In contrast, as little as 100 picograms of soluble Pep_{263} activated T lymphocytes (denoted with asterisk); up-regulation of the IL-2 receptor was two-fold higher than with control fractions. Substantial activation by Pep_{263} occurred on day 3, one day sooner than had been observed with whole organisms (see Figure 7). Pep_{263} bound to the microtiter plate or immobilized by linkage to protein A beads failed to activate T lymphocytes. These results show that Pep_{263} is capable of activating T lymphocytes to an extent that surpasses what we observed with whole *C. albicans* cells. Experiments to confirm that Pep_{263} expands the Vβ2 subset are included in this proposal.

Model for the Participation of Int1p in Candidemia

[00105] We have presented preliminary evidence that the *C. albicans* protein Int1p exerts superantigen-like effects on human T lymphocytes. Activation of T lymphocytes as measured by up-regulation of the IL-2 receptor (CD25) is not dependent upon antigen processing and presentation, can be blocked by antibodies to MHC Class II, and results in the expansion of the Vβ2 subset. Activation of T lymphocytes can be triggered by Pep_{263}, a 263 amino acid peptide

that is cleaved from the amino terminus of Int1p in a reaction accelerated by physiologic doses of heparin. Picogram inputs of Pep_{263} are equivalent to *INT1/INT1 C. albicans* or *INT1*-expressing *S. cerevisiae* in the ability to activate T lymphocytes. Like most microbial superantigens, Pep_{263} is active when soluble, not when bound to a microtiter plate or to antibody-coated beads.

[00106] Figure 16 schematizes the apparent role of Int1p in *C. albicans* fungemia. In the absence of heparin (panel A), the first 263 amino acids of Int1p (Pep_{263}) are covert and cannot be detected by anti-INT600 antibodies (Figure 12). Only in the presence of heparin (panel B) is the amino terminus of Int1p exposed, at which point Pep_{263} is cleaved and released into the fluid phase (panel C), where it exerts superantigen-like effects culminating in the release of pro-inflammatory cytokines that influence the clinical outcome. While it is possible that there are other superantigens liberated by *C. albicans*, or that even smaller fragments of Pep_{263} may also have superantigen-like effects, the activity of Pep_{263} and the applicability of these interactions may be applicable to the problem of candidemia in the NICU infant.